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Gonococcal Transferrin-Binding Protein 1 Is Required for Transferrin Utilization and Is Homologous to TonB-Dependent Outer Membrane Receptors

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The pathogenic *Neisseria* species are capable of utilizing transferrin as their sole source of iron. A neisserial transferrin receptor has been identified and its characteristics defined; however, the biochemical identities of proteins which are required for transferrin receptor function have not yet been determined. We identified two iron-repressible transferrin-binding proteins in *Neisseria gonorrhoeae*, TBP1 and TBP2. Two approaches were taken to clone genes required for gonococcal transferrin receptor function. First, polyclonal antiserum raised against TBP1 was used to identify clones expressing TBP1 epitopes. Second, a wild-type gene copy was cloned that repaired the defect in a transferrin receptor function (*trf*) mutant. The clones obtained by these two approaches were shown to overlap by DNA sequencing. Transposon mutagenesis of both clones and recombination of mutagenized fragments into the gonococcal chromosome generated mutants that showed reduced binding of transferrin to whole cells and that were incapable of growth on transferrin. No TBP1 was produced in these mutants, but TBP2 expression was normal. The DNA sequence of the gene encoding gonococcal TBP1 (*tbpA*) predicted a protein sequence homologous to the *Escherichia coli* and *Pseudomonas putida* TonB-dependent outer membrane receptors. Thus, both the function and the predicted protein sequence of TBP1 were consistent with this protein serving as a transferrin receptor.

The ability of many human pathogens to cause infection is dependent in part on their ability to acquire iron from their host. Unlike the members of the family *Enterobacteriaceae*, the pathogenic Neisseria species do not produce and secrete soluble siderophores to compete with host iron-binding proteins such as transferrin (Tf) and lactoferrin (Lf), but instead can acquire iron directly from these proteins (32, 33). Utilization requires direct contact between Tf and the cell membrane (1, 30, 53), which implies the presence of specific receptors for these iron-binding proteins. Recent evidence suggests that other human pathogens such as *Haemophilus influenzae* and *Bordetella pertussis* also possess specific receptors for Tf (43, 48).

Tf receptor function has been characterized in the pathogenic *Neisseria* species (8, 27, 50, 58). Tf utilization and binding is iron repressible (27, 50, 53, 60), suggesting the participation of one or more of the characterized iron-repressible outer membrane proteins (Frps) (14, 60) in receptor function. The receptor specifically recognizes human Tf and not Tfs from other mammalian sources (27, 50) and does not effectively discriminate between iron-loaded Tf and apotransferrin (8, 58). Competition experiments indicate the presence of discrete receptors for Tf and Lf. Both gonococcal and meningococcal Tf receptors are saturable at about 1 μM protein (8, 58), reflective of an estimated affinity that is approximately 500-fold lower than that of the human Tf receptor (59). The copy number of the bacterial receptor is 2,800 to 2,900 molecules per CFU (8, 58).

A set of genetically linked Tf receptor function (*trf*) mutants of *Neisseria gonorrhoeae* was recently described (8). These mutants were incapable of binding Tf or utilizing Tf-bound iron, while utilization of Lf was unaffected. Specific loss of Lf receptor function resulted from mutation of another locus, designated *lrf* (8). Another unlinked mutation (*tlu*) prevented Tf and Lf utilization without affecting ligand binding. The *tlu* mutation indicated the presence of a nonreceptor protein that was common to both Tf and Lf utilization pathways. The *trf*, *lrf*, and *tlu* gene products were not identified (8).

Two different meningococcal iron-repressible proteins (Frps) have been identified by their ability to bind human Tf and thus may be part of a Tf receptor. A 95-kDa Frp can be isolated from total membrane preparations by Tf affinity purification (49) and has been designated TBP1 (Tf-binding protein 1 [18, 39]). A smaller Frp, which varies in molecular mass (from 68 to 86 kDa) depending on the strain examined, is also isolated by Tf affinity purification (49). This protein, designated TBP2 (18, 39), retains its ability to bind Tf after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting (49, 50). To date, there is no conclusive evidence to indicate that either TBP1 or TBP2, together or singly, functions as the meningococcal Tf receptor. Little is known about Tf-binding proteins of the gonococcus. A TBP1 homolog was identified in a clinical gonococcal isolate (49); however, no gonococcal TBP2 homolog has been characterized.

In this study, we isolated a 100-kDa protein (TBP1) from total membrane preparations of gonococcal strain FA19 (33) by Tf affinity purification. We also identified an 85-kDa membrane protein analogous to meningococcal TBP2. Polyclonal antiserum raised against gonococcal TBP1 was used to screen an expression library for fragments of the gene encoding TBP1, *tbpA*. Simultaneously, part of a wild-type

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TABLE 1. Table of gonococcal strains

Strain	Genotype	Phenotype	Source or reference
FA19		Tf^+	33
FA6338	*trf-1*	Tf^-	8
FA6356	*trf-2*	Tf^-	8
FA6342	*trf-3*	Tf^-	8
FA6367	*trf-4*	Tf^-	8
FA6366	*trf-5*	Tf^-	8
FA6593	*trf-1*	Tf^-	This study
FA6747	*tbpA*∇*1*[a]	Tf^-	This study
FA6689	*tbpA*∇*2*[a]	Tf^-	This study

[a] ∇, transposon.

gene that repaired the defect in a *trf* mutant was cloned. DNA sequencing revealed that the clones obtained by these two different approaches overlapped. Introduction of transposons into these clones and then allelic replacement in the gonococcal chromosome generated mutants that could not grow on Tf as a sole iron source and bound reduced amounts of Tf to whole cells. They did not express TBP1, while TBP2 expression was normal. The predicted protein sequence of TBP1 was homologous to that of a class of gram-negative outer membrane receptors that depend on the function of TonB.

MATERIALS AND METHODS

Strains and growth conditions. All *N. gonorrhoeae* strains used in this study are described in Table 1.

Bacteria were routinely maintained on GCB agar (Difco) containing Kellogg's supplements I and II (23) in a 5% CO_2 atmosphere at 37°C. For Western blot (immunoblot) analysis of total membrane proteins, gonococci were iron stressed by growth in a chemically defined medium (CDM) which was made iron depleted by treatment with Chelex-100 (Bio-Rad) (60). Iron-replete controls were grown with 100 μM ferric nitrate. All glassware was acid washed to remove contaminating iron.

For large-scale preparation of total gonococcal membranes for the purpose of TBP1 purification, GCB broth medium containing Kellogg's supplement I was inoculated from 18-h-old, plate-grown, nonpiliated gonococcal cultures. After one mass doubling, the chelator Desferal (deferoxamine mesylate; CIBA Pharmaceutical Co.) was added to 50 μM to induce iron starvation. After 4 h of growth with the chelator, cells were harvested.

To determine a mutant's ability to grow on Tf, CDM agarose plates containing 25 μM Tf which had been 25% saturated with iron (33) were prepared. Mutants to be screened were streaked from GCB agar plates to CDM-Tf plates and allowed to grow at 37°C for 24 to 48 h in a 5% CO_2 atmosphere.

Preparation of total membranes. Cells were harvested, resuspended in 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (pH 7.4), and passed through a French pressure cell twice at 20,000 lb/in^2. Intact cells were removed by centrifugation at 8,500 × *g*, and membranes were pelleted at 140,000 × *g* for 1 h. The final membrane pellet was resuspended in 10 mM HEPES (pH 7.4), and the protein content was determined by a bicinchoninic acid assay (Pierce).

Polyclonal anti-TBP1 antiserum. Polyclonal antiserum was raised against gonococcal strain FA19 TBP1, which was purified from total membrane protein preparations by a procedure based on that of Schryvers and Morris (51) but with the following modifications. The batch purification was scaled up to accommodate 25 mg of total membrane proteins. Human Tf (Sigma) was biotinylated with NHS-S-S-biotin (Pierce) as described previously (51). The streptavidin-agarose affinity resin to which Tf and Tf-binding proteins were bound was washed with 50 mM Tris (pH 8.0)–1 M NaCl–10 mM EDTA–0.75% sodium lauroylsarcosine. Proteins were eluted from the affinity resin with 1% β-mercaptoethanol in the above washing buffer. Only TBP1 and Tf were recovered in this purification. These proteins were separated by preparative SDS-PAGE on 7.5% gels and stained with Coomassie blue (Fig. 1A). The 100-kDa protein band was excised from the gel; the acrylamide band was lyophilized, ground into a fine powder, and resuspended in phosphate-buffered saline. Approximately 500 μg of purified TBP1 mixed with Freund's complete adjuvant was injected subscapularly into female New Zealand White rabbits. The rabbits were boosted twice with the same amount of antigen in Freund's incomplete adjuvant at 2-week intervals. TBP1-specific antibodies were detected 2 weeks after the final immunization by Western blot against purified TBP1. The antiserum recognized only the 100-kDa protein species in total iron-stressed gonococcal membrane preparations (Fig. 1B).

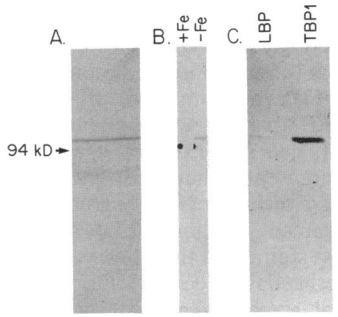

FIG. 1. Proteins recovered from Tf affinity purification and characterization of anti-TBP1 polyclonal antiserum. (A) Proteins isolated by Tf affinity included a 100-kDa protein and Tf, which migrated as a diffuse band at 85 kDa. Proteins were stained with Coomassie blue. (B) Western blot analysis of proteins from iron-replete (+Fe) and iron-starved (−Fe) total FA19 membranes probed with anti-TBP1 antiserum. (C) Western blot analysis of affinity-purified LBP and TBP1 probed with anti-TBP1 antiserum. Lanes in panel C were loaded equally with purified proteins. Position of 94-kDa molecular mass standard is indicated at left and pertains to all three panels.

Purification of gonococcal LBP. A 102-kDa Lf-binding protein (LBP) was isolated from iron-stressed total membrane preparations of FA19 by Lf affinity purification that was analogous to TBP1 purification except that human Lf (Sigma) was biotinylated with NHS-S-S-biotin (Pierce).

DNA isolation, digestion, and library construction. *Escherichia coli* plasmids were isolated, digested with restriction endonucleases, and used for cloning by standard methods (29). The chromosome of gonococcal strain FA19 was isolated and purified by CsCl gradient ultracentrifugation as described previously (55). The λgt11 expression library was constructed by sonication of FA19 DNA to sizes ranging from 0.5 to 1 kb in length; this was followed by *Eco*RI linker addition and ligation into λgt11 arms which had been digested with *Eco*RI. Ligated DNA was packaged with the Packagene kit manufactured by Promega. Bacteriophage were plated on *E. coli* Y1090 (Promega).

Two size-selected libraries were constructed for the pur-

pose of obtaining progressively larger fragments of the *tbpA* gene. Completely *Sau*3AI-digested FA19 DNA was size fractionated from 0.5 to 1.5 kb and then ligated into *Bam*HI-digested pUP1 (15). This library was transformed into $CaCl_2$-treated *E. coli* HB101 (29). A second library of 2.5- to 4.0-kb *Hin*cII fragments was generated by ligation of *Eco*RI linkers to *Hin*cII fragments and then ligation into λZAPII (Stratagene), which had been previously treated with *Eco*RI and alkaline phosphatase. The ligated DNA was packaged with the Packagene system, and the library was plated on *E. coli* DH5αMCR (Bethesda Research Laboratories).

Immunological screening of gonococcal λgt11 expression library. Primary anti-TBP1 antiserum was absorbed with an *E. coli*-phage lysate available from Stratagene according to the manufacturer's instructions. A 1:200 dilution of this absorbed antiserum was used to screen the λgt11/FA19 library following the protocol accompanying the Picoblue Immunological Screening Kit (Stratagene).

Cloning a DNA fragment capable of repairing the defect in *trf-1*. The library from which the *trf-1* mutant-repairing clone was isolated was constructed by ligation of partially digested, 0.5- to 6.0-kb *Sau*3AI fragments of FA19 DNA into *Bam*HI-digested pUP1. The library was transformed into library efficiency-competent DH5αMCR. A method similar to that described by Spratt (54) was used to identify progressively smaller pools of *E. coli* recombinants that could repair the *trf-1* defect. A *trf-1* derivative was constructed by transformation of the *trf-1* mutation by congression (8) into F62 (23). This strain, designated FA6593 (Table 1), was used in the following screening procedure since transformation efficiencies are reportedly higher when transforming DNA from *E. coli* into F62 (10). One thousand recombinant *E. coli* colonies were replica plated and pooled. Plasmid DNA was isolated (Qiagen, Chadsworth, Calif.) from each pool, and the DNA was transformed (5, 6) into FA6593. Tf^+ transformants were selected on CDM-Tf agarose plates. The screening procedure was repeated on smaller subsets of the original transforming pool until a single recombinant clone was identified that transformed Tf^- gonococci to Tf^+.

Transposon mutagenesis of gonococcal TBP1 clones. mTn*3* (Cm) transposons (52) were introduced into pUNCH403 and pUNCH115 (see Fig. 2) by using the shuttle mutagenesis technique described by Seifert et al. (52). Transposons were introduced into cloned gonococcal DNA in *E. coli*, and the exact locations were identified by sequencing the junctions with primers derived from the ends of the mTn*3*(Cm) transposon. DNA fragments into which transposons had been introduced were transformed (5, 6) into gonococcal strain FA19, and homologous recombination allowed replacement of the wild-type gene with the insertionally mutagenized gene.

Western blot analysis. Total membrane proteins (30 μg) were separated on 7.5% polyacrylamide gels (26) for anti-TBP1 immunoblots and horseradish peroxidase (HRP)-Tf blots. Proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell) in 20 mM Tris–150 mM glycine–20% methanol–0.1% SDS. For immunoblots with anti-TBP1 antibody, filters were blocked with 5% bovine serum albumin in 20 mM Tris (pH 7.5)–500 mM NaCl with 0.05% Tween 20 (TBS/T) for 1 h at room temperature. A 1:300 dilution of the antibody was allowed to bind for 1 h at room temperature, and this was followed by three 15-min washes with TBS/T. A 1:5,000 dilution of secondary antibody (goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate from Bio-Rad) was allowed to bind for 1 h at room temperature, and this was followed by three 15-min washes in TBS/T. Blots were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris (pH 9.5)–50 mM $MgCl_2$–100 mM NaCl. To detect TBP2, nitrocellulose filters to be probed with HRP-Tf were blocked in 50 mM Tris (pH 7.0)–150 mM NaCl (TBS) with 3% nonfat skim milk for 1 h at room temperature. HRP-Tf (Jackson ImmunoResearch) was added in TBS-skim milk to a concentration of 1 μg/ml and allowed to bind for 1 h at room temperature. Filters were washed twice for 15 min each in TBS/T and then once for 15 min in TBS and then exposed to high-sensitivity Enzygraphic Web (IBI).

DNA hybridization. For Southern blot analysis, chromosomal DNAs from FA19 and transposon mutants were digested with *Cla*I and fractionated on 0.7% agarose gels. Gels were transferred bidirectionally to Magnagraph nylon (Schleicher & Schuell). Blots were probed with random-primed, digoxigenin-dUTP-labeled DNA and developed according to the Genius labeling and detection kit instructions (Boehringer Mannheim Biochemicals). For library screening with DNA probes, the Genius labeling and detection system instructions were followed.

Whole-cell HRP-Tf binding assay. Gonococci were grown on CDM agarose plates containing either no added iron or 50 μM ferric nitrate for 24 h at 37°C with 5% CO_2. Cells were scraped from plates into CDM liquid medium containing no added iron, and 10^7 CFU were applied to nitrocellulose filters by using the Schleicher & Schuell dot-blot apparatus. Filters were probed with HRP-Tf and developed as described previously (8, 58).

DNA sequencing and sequence analysis. pUNCH405 and pUNCH115 were sequenced by using CsCl-purified, double-stranded plasmid template (24) and Sequenase by the dideoxychain termination method of Sänger et al. (44). Both strands were completely sequenced by using vector-specific or internal primers and both dG- and dI-labeling reactions. DNA sequence was analyzed with the Genetics Computer Group package (13) (University of Wisconsin). Pairwise alignments between TBP1 and TonB-dependent receptors were generated by the Bestfit program.

Nucleic acid sequence accession number. The gonococcal *tbpA* gene sequence presented in this report has been deposited with GenBank under the accession number M96731.

RESULTS

Gonococcal TBP1 purification and polyclonal anti-TBP1 antiserum characterization. Using a Tf affinity procedure that was developed for isolation of meningococcal TBP, a single gonococcal Frp of 100 kDa (TBP1) was isolated from total membrane preparations of strain FA19 (Fig. 1A). No gonococcal TBP2 was recovered in this purification. Antiserum raised against TBP1 recognized a single protein in iron-stressed total membrane preparations (Fig. 1B). This polyclonal antiserum cross-reacted weakly with affinity-purified LBP when equivalent amounts of LBP and TBP1 were loaded (Fig. 1C). LBP cross-reaction was also apparent in Western blots of total membrane proteins in which the amount of TBP1 compared with LBP was not constant (data not shown). The apparent molecular weight of LBP was slightly larger than that of TBP1. The polyclonal antibody also cross-reacted with 95- to 100-kDa protein species from meningococcal strain FAM2 (M986-NCV-1 [16]) and gonococcal strains MS11 (31), F62 (23), and UU1008 (obtained from Zell McGee, University of Utah) (data not shown). The antiserum did not, however, bind to the surface of intact

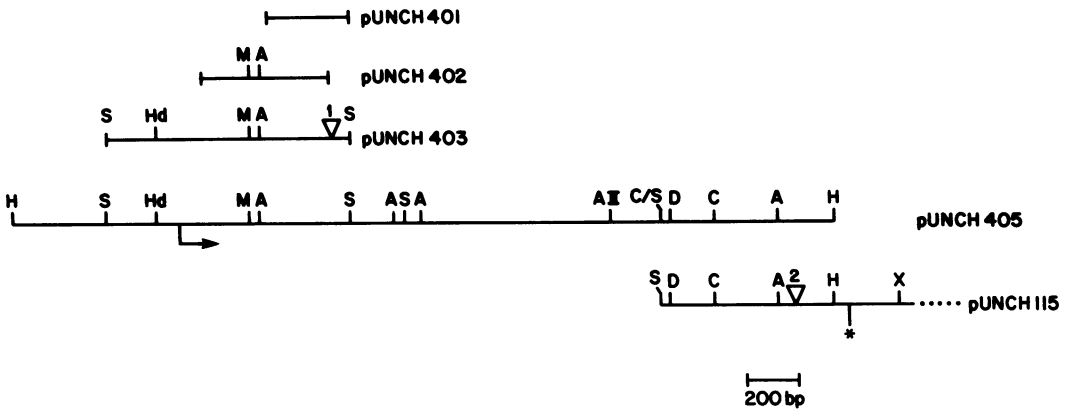

FIG. 2. Diagram of *tbpA*-specific clones. Symbol: ∇, positions of two transposons. ∇*1* is in strain FA6747, and ∇2 is in strain FA6689. Arrow indicates position of beginning and direction of open reading frame corresponding to *tbpA* gene. Asterisk indicates end of *tbpA* gene. The following designations indicate sites of restriction endonuclease recognition: A, *Ava*I; AII, *Ava*II; C, *Cla*I; D, *Dra*I; H, *Hin*cII; Hd, *Hin*dIII; M, *Mlu*I; S, *Sau*3AI; X, *Xba*I. C/S indicates that *Cla*I and *Sau*3AI sites overlap. The 2.8 kb downstream of the *Xba*I site in pUNCH115 are shown as a dotted line.

iron-stressed FA19 by whole-cell immunoblot assay (data not shown).

Cloning the *tbpA* gene by using anti-TBP1 antiserum as a probe. The polyclonal anti-TBP1 antiserum was used to screen an expression library of FA19 chromosomal DNA in λgt11. A total of 175,000 recombinant clones were screened, and the two antibody-reactive clones identified were further characterized. The polymerase chain reaction was applied to the λgt11 clones by using vector-specific primers, and the resultant polymerase chain reaction products were sized and subsequently cloned into the *Sma*I site of pBluescriptII (Stratagene). The smaller of the two clones was designated pUNCH401, and the larger was designated pUNCH402. These clones were subsequently shown by sequencing and restriction mapping to overlap (Fig. 2). Southern analysis of FA19 chromosomal DNA digested with *Sau*3AI, using pUNCH401 as a probe, identified a 1-kb *Sau*3AI fragment which was larger than but completely contained pUNCH401. Thus, pUNCH401 was used as a probe to screen a size-fractionated *Sau*3AI library (see Materials and Methods). Four identical clones were obtained, and one was further characterized and designated pUNCH403. This clone was sequenced and used for transposon mutagenesis (see below). Sequencing revealed that pUNCH403 overlapped pUNCH401 and pUNCH402 (Fig. 2). A library of size-fractionated *Hin*cII fragments in λZAPII was screened by using pUNCH401 as a probe. A 3.2-kb clone was isolated and named pUNCH405. The insert in pUNCH405 comigrated with a genomic *Hin*cII fragment identified by Southern blotting with pUNCH401 as a probe. Restriction mapping and sequencing (see below) revealed that pUNCH405 overlapped with pUNCH401, pUNCH402, and pUNCH403 (Fig. 2).

Southern blots of FA19 chromosome digested with *Ava*I, *Ava*II, *Cla*I, *Dra*I, *Hin*cII, *Mlu*I, and *Sau*3AI probed with each of the above clones identified a single chromosomal *tbpA* locus (data not shown).

Cloning a gene that repaired the defect in *trf-1*. Strain FA6593, which carried the *trf-1* mutation characterized in FA6338 (8), was transformed with pools of wild-type chromosomal DNA in a suicide vector, pUP1. Homologous recombination between the wild-type locus and the *trf-1* locus in the chromosome created transformants that were able to grow on CDM-Tf plates. Initially, five pools were screened for their ability to repair the Tf^- defect. Of these five pools, three were able to transform the Tf^- mutant to a Tf^+ phenotype at a surprisingly high frequency of ca. 10^{-4}/CFU. Smaller subsets of the original pools were screened, and ultimately, one clone was isolated which transformed the *trf-1* mutant to a Tf^+ phenotype at a frequency of 10^{-3}/CFU. The portion of the plasmid that was responsible for the Tf^+ phenotype was subcloned into pBluescriptII and named pUNCH115. The insert in this clone was 3.7 kb and by DNA sequencing was shown to overlap pUNCH405 (Fig. 2). Subsequent analysis confirmed that pUNCH115 contained the 3′ end of the *tbpA* gene and that pUNCH405 contained the 5′ end of the same gene.

Transposon mutagenesis of *tbpA* clones. To determine the effect of inactivation of the *tbpA* gene, pUNCH403 and pUNCH115 were insertionally mutagenized, using the mini-transposon mTn*3*(Cm). Four transposon insertions in different positions within the open reading frame in pUNCH403 were characterized and, after recombination into the FA19 chromosome, were shown to be identical in phenotype (see below). The position of insertion and phenotypes of one representative transposon mutant will be described in detail here. Similarly, one transposon insertion in pUNCH115 was further characterized. As indicated in Table 1, the gonococcal strain carrying the transposon insertion in position ∇*1* (Fig. 2) was designated FA6747, and the strain carrying the transposon insertion in position ∇2 (Fig. 2) was designated FA6689. Neither FA6747 nor FA6689 grew on CDM plates supplemented with Tf (Fig. 3). The *trf-1* mutant (FA6338), which was repaired by pUNCH115, also could not grow on Tf (Fig. 3). In contrast, strains FA6338, FA6747, and FA6689 grew as well as wild type on CDM supplemented with Lf. Neither the transposon mutants FA6747 and FA6689 nor the *trf-1* mutant FA6338 were capable of uptake of iron from Tf, while iron uptake from Lf was unchanged (data not shown).

TBP1 and TBP2 expression in *trf-1* and transposon insertion mutants. Membranes from strains FA6338, FA6747, and FA6689 as well as the wild-type strain FA19 were isolated and tested for the presence of TBP1 and TBP2 (Fig. 4). The 100-kDa TBP1 protein was only present in iron-stressed membranes of FA19. Neither the original *trf-1* mutant,

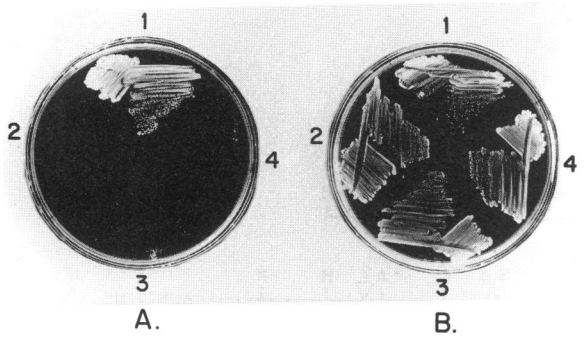

FIG. 3. Growth phenotypes of *trf-1* and transposon insertion mutants. (A) Plate containing Tf; (B) plate containing Lf. Quadrant 1 was streaked with FA19, 2 with FA6338 (*trf-1*), 3 with FA6747 (*tbpA*∇*1*), and 4 with FA6689 (*tbpA*∇*2*).

FA6338, nor the transposon mutants, FA6747 and FA6689, expressed any detectable TBP1 (Fig. 4A). Expression of TBP2, an 85-kDa iron-repressible protein, was unaffected (Fig. 4B). The inability to express TBP1 and the ability to express TBP2 were shared by the other previously described (8) *trf* mutants, FA6356, FA6342, FA6367, and FA6366 (data not shown). Expression of TBP1 and TBP2 was normal in all the previously isolated (8) *lrf* and *tlu* mutants (data not shown). The previously characterized *trf* mutants (8) were all repaired by an intact *tbpA* gene, which was constructed by ligation of the insert in pUNCH405 with the insert in pUNCH115 at the *Cla*I site (data not shown).

Southern blot analysis of transposon insertion mutants. To ensure that the transposon had recombined into the expected locus in the FA19 chromosome, DNA from the transposon mutants and FA19 was digested and probed with either a *tbpA*-specific probe or an mTn*3*(Cm)-specific probe. The transposon in strain FA6747 (*tbpA*∇*1*) caused a shift in the molecular weight of the 7.8-kb *Cla*I fragment resulting in a 9.4-kb band which hybridized to the mTn*3*(Cm)-specific probe (Fig. 5, lane 2). Similarly, the transposon insertion in strain FA6689 (*tbpA*∇*2*) caused a shift in the molecular weight of the 3.5-kb *Cla*I fragment, resulting in a 5.1-kb band which hybridized to the mTn*3*(Cm) probe (Fig. 5, lane 3). Thus, both transposon insertions recombined into the appropriate chromosomal locus without concomitant rearrangement.

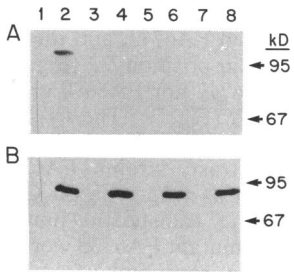

FIG. 4. TBP1 and TBP2 expression by *trf-1* and transposon insertion mutants. (A) Western blot probed with anti-TBP1 antiserum. (B) Ligand-binding Western blot probed with HRP-Tf. Lanes contain the following four strains: 1 and 2, FA19; 3 and 4, FA6338 (*trf-1*); 5 and 6, FA6747 (*tbpA*∇*1*); 7 and 8, FA6689 (*tbpA*∇*2*). Odd-numbered lanes contain iron-sufficient strains; even-numbered lanes contain iron-starved strains. Positions of molecular mass standards are indicated at the right in kilodaltons (kD).

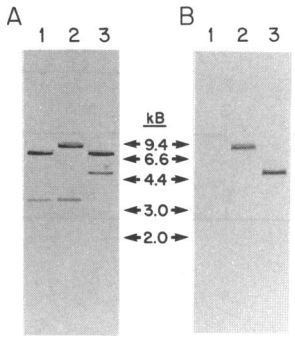

FIG. 5. Southern blot analysis of transposon mutants. (A) Southern blot probed with *tbpA*-specific probe, pUNCH405. (B) Southern blot probed with mTn*3*(Cm)-specific probe. Lanes contain chromosomal DNA from the following strains: 1, FA19; 2, FA6747 (*tbpA*∇*1*); 3, FA6689 (*tbpA*∇*2*). The pUNCH405 probe contains two *Cla*I sites, while the mTn*3*(Cm) probe has no internal *Cla*I sites. The pUNCH405 probe hybridized to three chromosomal *Cla*I fragments (7.8, 3.5, and 0.2 kb), and the mTn*3*(Cm) probe hybridized to one fragment. The 0.2-kb hybridizing fragment is not shown in this figure. The size of the mTn*3*(Cm) transposon was 1.6 kb. Positions of molecular size standards are indicated in kilobases (kB).

HRP-Tf binding to whole cells of *trf-1* and transposon insertion mutants. The HRP-Tf binding assay measures Tf receptor function at the cell surface (8). Whole cells of iron-starved FA19 bound approximately four- to sixfold more HRP-Tf than iron-sufficient FA19 (Fig. 6). All the mutants bound 4- to 6-fold less HRP-Tf than wild type when iron starved and 8- to 10-fold less HRP-Tf than wild type when iron sufficient (Fig. 6). All TBP1 mutants exhibited significant, and approximately equal, iron-repressible Tf binding compared with *E. coli*, which bound no Tf in the same assay (Fig. 6 and data not shown).

DNA sequencing and analysis of predicted protein sequence. The sequence of the gonococcal *tbpA* gene is presented in Fig. 7, from the *Hin*dIII site in pUNCH405 through the putative rho-independent termination structure, indicated by the inverted horizontal arrows, in pUNCH115. The boxed regions near the 5′ end of the gene indicate putative −10 and ribosome-binding sites. No canonical −35 sequence was identified in the hypothetical promoter region. A consensus Fur-binding site (2) was also notably absent from the *tbpA* promoter. This sequence has been shown to bind the Fur (ferric uptake regulator) repressor and to overlap the promoter region of iron-regulated genes (2). No evidence was

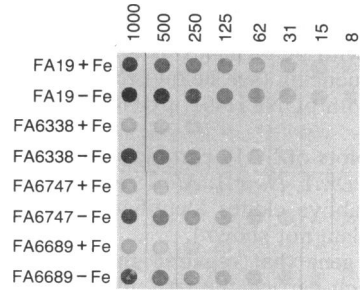

FIG. 6. Binding of HRP-Tf to iron-replete (+Fe) and iron-stressed (−Fe) whole cells. Strain and iron condition are indicated at the left, and the concentration of HRP-Tf (nanograms per milliliter) is shown above each column.

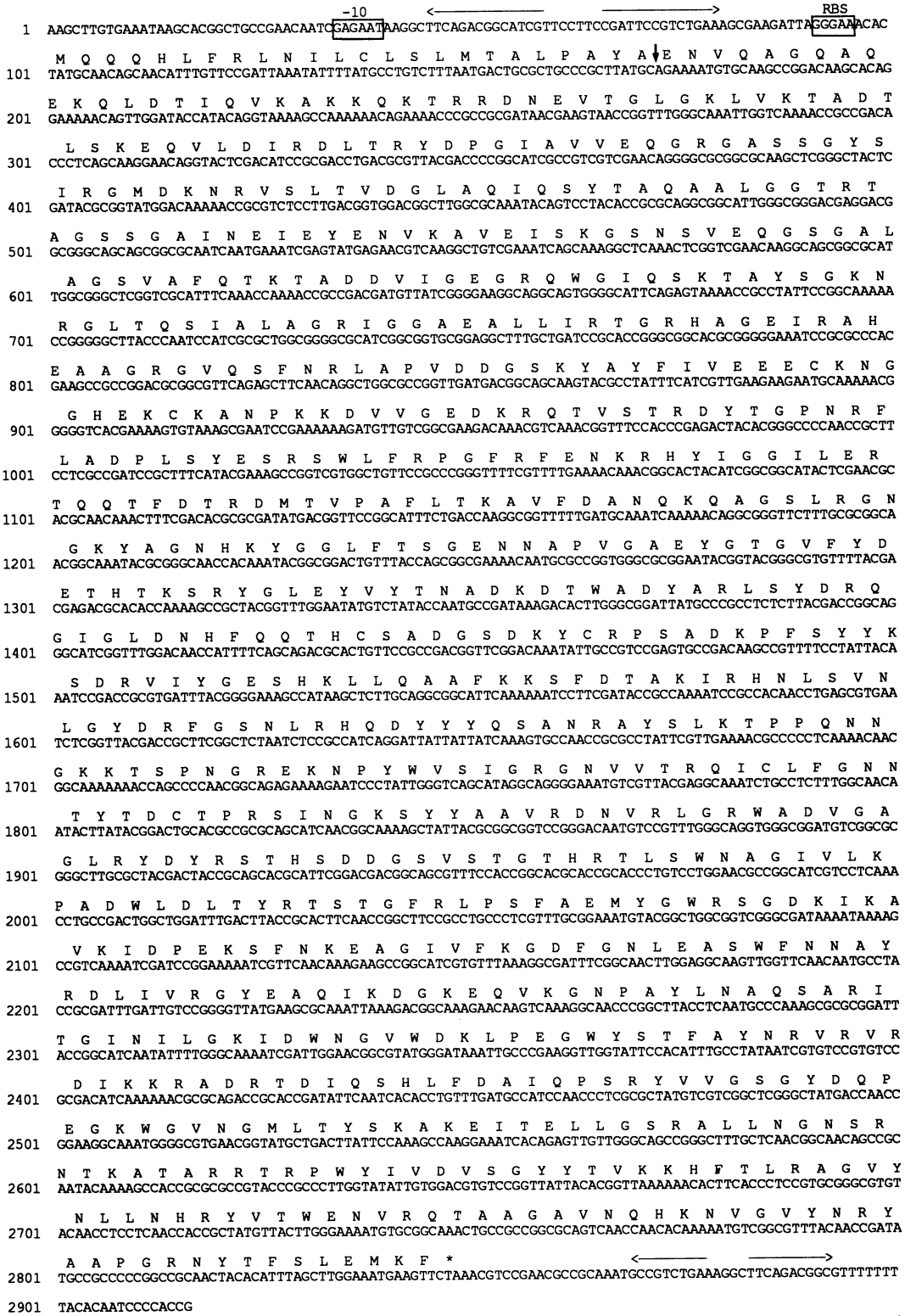

FIG. 7. Nucleotide sequence of the gonococcal *tbpA* gene. Single-letter codes for the deduced amino acid sequence are shown above the nucleotide sequence. Asterisk indicates the termination codon. Inverted arrows indicate inverted repeat regions capable of forming stem-loop structures. Putative −10 and ribosome-binding sites (RBS) are boxed. Vertical arrow indicates putative cleavage site between signal and mature peptides.

found of additional promoter sequences or Fur-binding sites in the DNA sequence extending 305 bp upstream of the *Hin*dIII site (data not shown). Between the putative −10 and ribosome-binding sites was an inverted repeat region capable of forming a secondary structure with a calculated free energy of −23.6 kcal at 25°C (calculated according to reference 57). This sequence is indicated by horizontal inverted arrows. The inverted repeat at the 3′ end of the *tbpA* gene had a calculated free energy of −16.5 kcal at 25°C and had characteristics of a rho-independent transcription terminator (61). Gonococcal uptake sequences (15, 17) were found within the inverted repeat structures at both ends of the *tbpA* gene.

The calculated molecular weight of the deduced amino acid sequence of TBP1 was 102,212, which agreed well with polyacrylamide gel estimates of 100,000. The calculated isoelectric point of TBP1 was 10.38, making it a very basic protein. The vertical arrow near the amino terminus of the predicted protein sequence indicates the putative cleavage site between the mature protein and the signal peptide. This cleavage site, which matched well with the *E. coli* consensus for signal peptidase cleavage sites (38), was determined by homology with the meningococcal (FAM20 [14]) TBP1 sequence, for which the amino-terminal sequence of the mature protein was determined (40). Of the amino-terminal nine residues that were determined (40), seven were identical to the predicted gonococcal protein sequence. The mature protein ended in a phenylalanine residue, which was preceded by alternating hydrophilic and hydrophobic residues for the next 10 amino acids. This pattern has been suggested to be important for outer membrane localization (56). A search of translated GenBank sequences yielded no significant matches beyond localized homology with the *E. coli* TonB-dependent outer membrane receptors (see below). A direct sequence comparison of the human Tf receptor (46) and TBP1 indicated that the two proteins were not related.

Identification of homology between TBP1 and TonB-dependent outer membrane receptors. A search of translated GenBank sequences identified localized homology between TBP1 and the *E. coli* TonB-dependent outer membrane receptors. These receptors depend on the function of TonB, which is thought to be necessary for energy transduction from the cytoplasmic membrane to energy-requiring outer membrane processes (41). An alignment of *E. coli* TonB-dependent outer membrane receptors was provided by R. Kadner (University of Virginia). Then pairwise alignments between TBP1, the *Pseudomonas putida* pseudobactin receptor PupA, and the *E. coli* receptors were generated by the Bestfit program (Genetics Computer Group package). All seven regions of homology identified by Kadner (22) were also identified in TBP1. These homologous domains and the extent of their amino acid similarity are shown in Fig. 8. Domains 1, 3, and 6 in PupA were previously identified (7); however, this is the first demonstration of PupA alignment in the other domains. Domain 1 includes the so-called "TonB box," which has been implicated in the direct interaction between TonB and these outer membrane receptors (3, 9, 20, 47). Domain 7 includes the motif suggested by Struyvé et al. (56) to be important in outer membrane localization.

DISCUSSION

The precise functions of TBP1 and TBP2 in iron acquisition are unclear. Since both proteins are iron repressible and bind to Tf under different experimental conditions both, working either in complex or independently, have been considered candidates for the Tf receptor (49, 50). In this study, we cloned the gene encoding gonococcal TBP1 (*tbpA*) and demonstrated that mutations in this gene caused total inability to internalize iron from Tf and to grow on Tf as the sole iron source. These phenotypes indicated that TBP1 was essential for iron acquisition from human Tf. The *tbpA* mutations caused a reduction in the ability of whole cells to bind Tf, but significant HRP-Tf binding remained. The residual HRP-Tf binding might be due to TBP2 or to another receptor, the presence of which was not indicated by Scatchard analysis (8).

Cloning the structural gene for TBP1 from an expression library by using polyclonal antiserum required purification of TBP1. Both gonococcal (this study) and meningococcal (49, 50) TBP1s bind to Tf only in affinity purification in which the starting material is membrane fragments. All efforts to reconstitute Tf binding by TBP1 after SDS-PAGE and electroblotting have failed (49, 50). This may indicate that ligand binding to TBP1 requires the maintenance of a particular protein conformation in the membrane that SDS-PAGE destroys. Tf binding by meningococcal TBP2 can be demonstrated both in membrane fragments and after denaturation, which has led to speculation that TBP2 is the strongest candidate for the Tf receptor (50). We were unable to purify TBP2 from gonococci by Tf affinity procedures using both high-salt (Fig. 1) and low-salt (data not shown) washes, although TBP2 was readily demonstrable by ligand-binding Western blotting. Reasons for the failure to isolate TBP2 from gonococci by affinity procedures are unclear.

The polyclonal antiserum raised against TBP1 was cross-reactive against the TBP1s from three other gonococcal strains and one meningococcal strain. Antibodies raised against denatured protein are likely to recognize some non-surface-exposed epitopes which may be buried in the membrane. These non-surface-exposed epitopes may be less variable, and thus antibody directed against them might be expected to be more broadly cross-reactive. The anti-TBP1 antiserum also cross-reacted to a slight extent with LBP. This may be due either to these proteins sharing common epitopes or to slight contamination of the TBP1 purification with LBP to which a subpopulation of antibodies was then raised.

Five linked *trf* mutants were previously characterized (8) and shown to be deficient in Tf receptor function. It was unclear from the previous study (8) whether the *trf* mutations directly affected the Tf receptor or products necessary for assembly or stability of the functional receptor. We cloned a wild-type DNA fragment that repaired the *trf-1* mutation and showed that it was a portion of the *tbpA* gene. Thus, *trf-1* was the result of a *tbpA* mutation. All the other chemically derived *trf* mutants (8) had a $TBP1^-$ $TBP2^+$ phenotype identical to that of the transposon mutants described here (Fig. 4 and data not shown). Subsequently, we found that each of the other independently isolated and characterized *trf* mutants (8) could be repaired by the cloned wild-type *tbpA* gene, indicating that all these mutations were within the structural gene encoding TBP1. These data indicate that alteration of TBP1 is considerably more common than alteration of TBP2 among mutants selected for inability to use Tf as an iron source. We cannot conclude, however, that TBP2 is not involved in formation of a functional Tf receptor or in iron uptake from Tf. Specific mutagenesis of TBP2 will be required to answer these questions.

The surprisingly high frequency (ca. 10^{-3}) at which the cloned *tbpA* gene was able to transform Tf^- gonococci to Tf^+ might be due to the close proximity of the DNA uptake sequence found at the 3′ end of the *tbpA* gene. Our inability

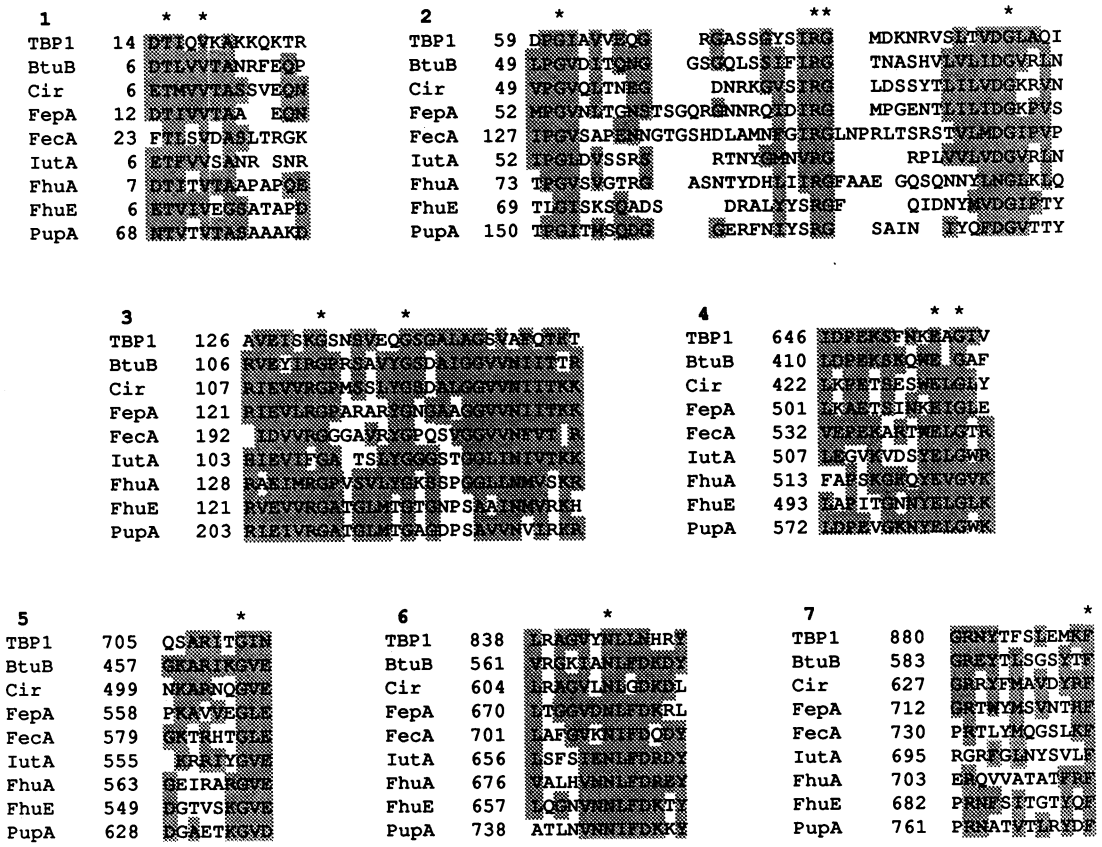

FIG. 8. Peptide alignment between TonB-dependent outer membrane receptors of *E. coli* and *P. putida* and TBP1. Alignment of sequences currently posted to GenBank includes TBP1; BtuB (*E. coli* vitamin B_{12} receptor [19]); Cir (*E. coli* colicin I receptor [35]); FepA (*E. coli* ferric enterochelin receptor [28]); FecA (*E. coli* ferric citrate receptor [42]); IutA (*E. coli* aerobactin receptor [25]); FhuA (*E. coli* ferrichrome receptor [12]); FhuE (*E. coli* coprogen and rhododuric acid receptor [45]); PupA (*P. putida* pseudobactin receptor [7]). Numbers following name of the protein indicate positions of first amino acid of homologous peptide in mature protein. Asterisks indicate positions of complete identity in alignment; shaded regions denote similar amino acid residues.

to repair *lrf* and *tlu* mutations using a similar procedure (data not shown) might indicate that the *trf-1* mutation was particularly amenable to cloning by repair.

The mechanism by which iron concentration regulates gene expression has been described in detail for *E. coli* (2). The consensus Fur-binding site overlaps the promoter of iron-regulated genes. Iron is a corepressor such that at high intracellular concentrations of iron, the Fur-iron complex binds and prevents transcription. Several iron-repressible proteins have been identified in *Neisseria* species, and one iron-repressible promoter has been characterized (34). This promoter, which precedes the gonococcal *fbp* (ferric-binding protein) gene, contains an *E. coli* consensus Fur-binding site and is thus suggested to be regulated in a fashion similar to iron-regulated genes in *E. coli* (2). The putative *tbpA* promoter, in contrast, was atypical in that neither −35 nor Fur-binding site consensus sequences could be identified. Another novel feature in the *tbpA* promoter was the long inverted repeat between the putative −10 and ribosome-binding sites. The roles of inverted repeats and their ability to form secondary structures have also been recognized as important features of gene regulation (21, 61). Identification of the *tbpA* promoter is speculative in the absence of transcriptional start site data. At present, the mechanism by which *tbpA* expression is regulated is unclear.

The sequence of the *tbpA* gene strongly suggests that this protein serves as a Tf receptor. First, the presence of a signal sequence indicates that it probably functions beyond the cytoplasmic membrane. Second, seven homologous domains within the *E. coli* TonB-dependent outer membrane receptors were also found in the sequence of TBP1. Homology with these proteins indicated that TBP1 is a member of a class of proteins that serve as specific receptors for necessary nutrients such as iron-siderophore complexes and vitamin B_{12}. The first homologous domain has been implicated in direct protein-protein interaction with TonB (3, 9, 20, 47). Domain 7 contains an amphipathic motif preceding a terminal phenylalanine residue. This pattern has been recognized as common among virtually all outer membrane proteins and was suggested to be essential for outer membrane localization (56). The biological significance of the other homologous regions is unknown. They may be required for TonB interaction or may be necessary for outer membrane localization and/or stability. In any event, the homology between these receptors and TBP1 argues that TBP1 is a TonB-dependent outer membrane receptor and furthermore that *Neisseria* species, as has been suggested for all gram-negative bacteria (41), have a TonB homolog.

Other similarities exist between TBP1 and the *E. coli* TonB-dependent receptors. Many of the TonB-dependent receptors are iron regulated and are involved in iron utilization (7, 12, 25, 28, 42, 45), as is true for TBP1. One difference

is that the *E. coli* outer membrane proteins are acidic, with pIs in the range of 4 to 5.5, whereas TBP1 is very basic. Other neisserial proteins which are basic include Fbp (4) and the Opa proteins (36). It has been suggested that basic surface proteins are beneficial in vivo for intracellular pathogens by inhibition of phagolysosomal fusion (11).

Interesting questions remain concerning how the neisserial Tf receptor functions in vivo. The neisserial Tf receptor has an estimated affinity which is approximately 500-fold lower than that of the human Tf receptor (8), although technical considerations suggest that the affinity of the bacterial receptor might have been underestimated (58). One wonders how a low-affinity bacterial receptor could compete with its human counterpart in vivo. Since the concentration of Tf in serum (59) is about 30-fold higher than that required to saturate the neisserial Tf receptor, competition with the human receptor might not be necessary. Since the neisserial receptor recognizes only human Tf and the human receptor recognizes the Tfs from several mammalian sources (37), the eukaryotic and prokaryotic receptors may recognize different domains of Tf. There are other obvious differences in the receptors, including internalization of the Tf receptor complex in eukaryotes (59), whereas *Neisseria* species do not internalize Tf (1, 30, 53). Also, anti-human and anti-chicken Tf receptor antibodies do not recognize the surface of *Neisseria* species (8). These contrasts suggest that these receptors are quite different, and in fact, the sequence analysis of TBP1 compared with the human Tf receptor confirms this hypothesis.

In summary, we cloned the gene encoding gonococcal TBP1 and showed that this protein is essential for growth on human Tf. The predicted amino acid sequence of TBP1 is homologous not to that of the eukaryotic Tf receptor but to those of other bacterial TonB-dependent outer membrane receptors. Thus, both the function and predicted sequence of TBP1 strongly suggest that this protein is a gonococcal Tf receptor.

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